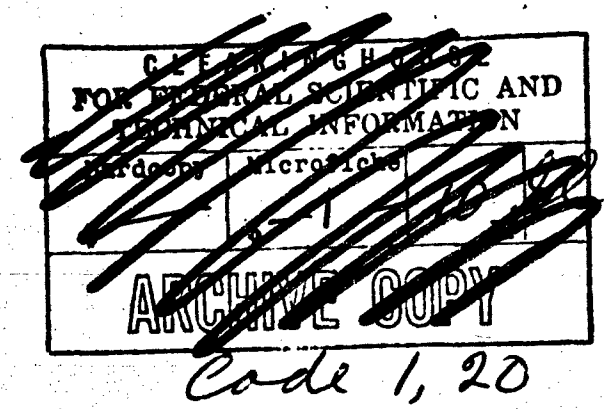

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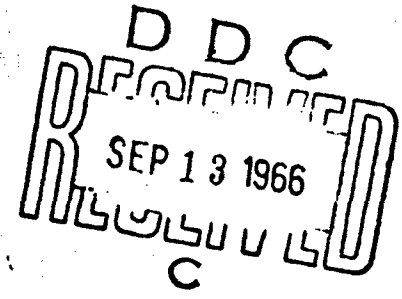

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Quantitative Assay for Crude Anthrax Toxins

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ABSTRACT

HAINES, BERTRAM W. (U.S. Army Biological Laboratories, Frederick, Md.), FREDERICK KLEIN, AND RALPH E. LINCOLN. Quantitative assay for crude anthrax toxins. J. Bacteriol. 89:71–85. 1965.—The whole crude toxins of *Bacillus anthracis*, although apparently responsible for the death of animals with anthrax, had never been quantitated. A total of 11 lots of the toxic culture filtrate of *B. anthracis* were pooled into one large lot of crude anthrax toxins. An extensive assay of this reference material was conducted in four laboratories by use of the time to death of the intravenously challenged Fischer 344 rat as the response variable. Doses of the material were varied factorially by concentration, dilution, and volume. The data from this study were used to define a potency unit of the crude anthrax toxins. Procedures were developed and illustrated for the assay of unknown lots of the toxins by comparing the rat time-to-death response to the unknown with either (i) the responses reported in this study, or (ii) directly with the rat responses to a new sample of the reference toxins. The possibilities and limitations of this standardization and of the statistical procedure through which it was developed are discussed.

The excellent work of Smith, Keppie, and Stanley (1955*a*), demonstrating the toxins of *Bacillus anthracis* organisms in the blood from guinea pigs in the terminal stages of anthrax, rekindled interest in the disease, particularly its toxins. (The toxic metabolic by-products of the growth of *B. anthracis* are composed of components with different biological or chemical properties. Naturally produced combinations of these components in unknown proportions will be referred to in this paper as "toxins.") To date, valid comparisons of results among the several experimenters (Smith et al., 1955*a*, *b*, 1956; Smith and Gallop, 1956; Thorne, Molnar, and Strange, 1960; Stanley and Smith, 1961; Beall, Taylor, and Thorne, 1962; Klein et al., 1962; Keppie, Smith, and Harris-Smith, 1955; Eckert and Bonventre, 1963; Harris-Smith, Smith, and Keppie, 1958; Sargeant, Stanley, and Smith, 1960; Stanley, Sargeant, and Smith, 1960) who have reported work with the toxic materials produced by *B. anthracis* have been difficult, because either whole crude toxins or the several components have been assayed by different methods, in different assay animals, and with no reference standard of the toxins.

This paper presents the results of studies to quantitate, in terms of defined potency units, the lethality of anthrax toxins in Fischer 344 rats. The authors developed a reference lot of stabilized freeze-dried crude anthrax toxins. This reference material was used in the study described here, and is available for other studies against which samples of anthrax toxins of unknown concentration can be assayed.

MATERIALS AND METHODS

Animals. Fischer 344 albino rats weighing 200 to 300 g were obtained from the Fort Detrick colonies of Frank Beall and Frederick Klein. Both colonies are maintained through brother-sister matings descended from the colony described by Taylor, Kennedy, and Blundell (1961). This weight range was chosen, because preliminary data indicated that the response time of rats that weigh more than 300 g was significantly greater than that of rats weighing more than 200, but less than 300, g. Further study on rats, carefully selected for weight, revealed no significant difference within the weight range of 200 to 300 g (Table 1). The analysis of variance is presented in Table 2.

Rat lethal test. Toxins of *B. anthracis* were injected into the dorsal vein of the penis of the Fischer rat. In describing this test, Beall et al. (1962) noted a definite relationship between the dose of the toxins injected and time-to-death.

Antiserum. Equine hyperimmune serum (DH-1-6C), prepared by repeated injections of spores of the Sterne strain of *B. anthracis*, was used (Thorne et al., 1960).

Preparation of anthrax toxins. The medium used was described by Thorne et al. (1960), and was made with triple-distilled water. Subsequent to his original description, Thorne (*personal communication*) has suggested some changes. The medium used in this study was as follows.

Nine stock solutions (A, B, C, D, E, F, G, H,

TABLE 1. *Response time in minutes of 27 rats injected with 1 ml of crude anthrax toxins by weight of rat*

Weight (g) of rat		
200	250	300
99	102	100
97	81	94
96	80	88
94	79	105
93	78	90
92	114	101
89	76	78
88	102	82
87	71	86
835*	783	824
92.6†	84.9	90.7

* Totals.
† Harmonic means.

TABLE 2. *Analysis of variance of reciprocal response times recorded in Table 1*

Source	df*	Sum of squares	Mean square	F
Between weights	2	.0485	.0242	1.50*
Within weights	24	.3859	.0161	
Total	26	.4344		

* Degrees of freedom.
† Not significant.

and I) were prepared. All stock solutions may be stored at 4 C for indefinite periods of time. Solution A contained $CaCl_2 \cdot 2H_2O$, 0.368 g/500 ml of water; B contained $MgSO_4 \cdot 7H_2O$, 0.493 g/500 ml of water; C contained $MnSO_4 \cdot H_2O$, 0.043 g/500 ml of water; D contained adenine sulfate, 0.105 g, and uracil, 0.070 g (both solids were dissolved in 100 ml of water, and the total volume was made up to 500 ml).

Solution E contained thiamine HCl, 0.025 g/500 ml of water; F contained tryptophan, 2.000 g; cystine, 0.600 g; and glycine, 0.750 g. The solids in solution F were dissolved as follows. Tryptophan was dissolved in 6 ml of 6 N HCl. Cystine was dissolved in 100 ml of water. Glycine was dissolved in 150 ml of water. These three solutions were combined, and water was added to bring the total volume up to 500 ml.

Solution G contained KH_2PO_4, 34.0 g/500 ml of water; H contained K_2HPO_4, 43.6 g/500 ml of water; I contained charcoal (Norit A), 3.75 g/500 ml of water.

A 10-ml amount of each stock solution, except that containing charcoal, was added to a suitable container; and 3.6 g of Casamino Acids (Difco) were added. The volume was brought up to 1 liter with triple-distilled water, and the *p*H of the medium was adjusted to 6.9 with 1 N H_2SO_4 or 1 N NaOH as needed. A 460-ml amount of this preparation was dispensed into a 3-liter Fernbach flask; 2 ml of charcoal suspension were added, and the preparation was autoclaved for 20 min at 15 psi.

Inoculation procedure. A 5-ml amount of 20% glucose (sterilized by filtration) was added to the Fernbach flask containing 460 ml of sterilized basal medium. Each flask of final medium was inoculated with 2×10^6 Sterne strain spores. The inoculated flasks were incubated statically for 23 to 27 hr at 37 C; 4 hr after inoculation 55 ml of 9% $NaHCO_3$ were added to each flask.

This final culture was centrifuged at 3,000 $\times$ *g* for 30 min. The supernatant fluid was decanted, and 10% horse serum was added. The solution was then sterilized by filtration through an ultrafine glass filter.

A preliminary test, to determine the potency of each of 14 toxic filtrates, was done by injecting 1-ml samples of each filtrate intravenously into two rats. The response (death) times of the rats were considered as indications of the toxicity of each batch. The total volume per batch and the response times of the test rats are given in Table 3.

The 14 toxic filtrates were combined, and a second preliminary test was conducted on the pooled material. The two rats used in this test died in 104 and 117 min, with a mean response time of 110.5 min. Both response times are within one standard deviation of the mean of all batches.

The pooled toxins were dispensed into 600 drying ampoules (40 ml), each containing 10 ml of

TABLE 3. *Volume per batch and response time of rats challenged with toxins by batch*

Batch	Total volume	Response time (min)		
		Rat A	Rat B	Mean
	ml			
1	450	97	92	94.5
2	450	107	91	99.0
3	450	97	96	96.5
4	400	95	—*	95.0
5	420	122	124	123.0
6	450	114	125	119.5
7	510	116	90	103.0
8	410	121	120	120.5
9	370	88	82	85.0
10	510	90	94	92.0
11	465	106	94	100.0
12	425	106	92	99.0
13	425	117	121	119.0
14	300	100	117	108.5
Total	6,005			103.9†

* Missed the vein.
† SD = 12.14.

toxins. Ampoules were shell-frozen in Dry Ice and alcohol (−79 C). Frozen ampoules were placed on an Amineo Dryer (American Instrument Co., Silver Spring, Md.), and dried under vacuum of 10 to 30 μ of mercury for 18 to 24 hr. Ampoules were sealed under vacuum, packed in cardboard containers, and stored at −20 C. A third preliminary test was conducted at this point. One randomly selected ampoule was reconstituted with 10 ml of triple-distilled water. A 1-ml amount of this toxic material was assayed in each of five rats. Their mean response time was 117.2 min. To further test the toxicity, 0.2 ml of undiluted and of serial twofold dilutions of the reconstituted material was injected intradermally into the shaven sides of a guinea pig, and observed for edematous reaction. The material reacted at a dilution of 1:32, and can be expressed according to Thorne et al. (1960) as containing 32 toxic units. Additional vials were reconstituted to 4× concentration, and tested on immunodiffusion plates against the standard spore antiserum (Thorne et al., 1960). Three individual lines of precipitate appeared in parallel arrangement when tested with a linear pattern. The strongest precipitate line was identified as the protective antigen (factor II) component when compared with a standard (Beall et al., 1962). An undiluted sample of the resuspended material had a protective antigen titer of 1:64 against the standard spore antiserum.

Reference toxins. These preliminary tests constituted quality control measures on the remaining 597 vials of dried toxic filtrate. As a result of these tests, it was known that these vials contained the known components of anthrax toxins.

Procedures. The toxins were assayed independently by each of four investigators. The procedures followed by each of the four were as similar as possible.

The characterization of the dose-response relationship of the toxins in Fischer rats was based on an assay in which the two dose factors of amount and concentration of toxins were each tested at several levels as follows: (i) five levels of the amount of toxins designated as 4 ml, 2 ml, 1.5 ml, 1 ml, and 0.5 ml; (ii) seven levels of the concentration of the toxins designated as 4×, 2×, 1×, 0.5×, 0.25×, 0.125×, and 0.0625×, where 1× is defined as the concentration resulting when 1 ampoule is reconstituted to 10 ml with a diluent of triple-distilled water. Dilutions beyond 1× were made with distilled water plus 10% normal horse serum.

The 7 × 5 factorial combinations of the several levels of these two factors, plus 19 control groups, were each tested in two Fischer rats by each of four investigators (Table 4). Three sets of control animals are not shown in Table 4. The first set included five pairs of rats. Each pair was inoculated with one of the five amounts of diluent alone (i.e., triple-distilled water plus 10% normal horse serum) to provide assurance that their companion animals responded to toxins as opposed to the inoculation of the diluents. The second set included seven pairs of animals. Each pair in this set was inoculated with 1.5 ml of one of the seven concentrations of toxins mixed with 0.5 ml ($\frac{1}{3}$ by volume) of specific antiserum (Thorne et al., 1960). The seven pairs of animals in the third set of controls were inoculated with 1.5 ml of one of the seven concentrations of toxins mixed with 0.5 ml of normal horse serum. These animals provided assurance that the control no. 2 animals that lived were saved by the antiserum specific against anthrax toxins.

Each investigator required 32 ampoules of dried toxins. Each of the 32 ampoules was opened, and reconstituted with 2.5 ml of diluent precooled to 4 C. The contents of all 32 ampoules were then pooled, providing a total of 80 ml of reconstituted

TABLE 4. *Response times in minutes of 280 Fischer rats by dose, concentration, technician, and rat*

Concn	Technician	4*		2*		1.5*		1*		0.5*	
		A†	B	A	B	A	B	A	B	A	B
4×	1	58	55	53	54	57	57	61	60	76	71
	2	53	61	54	52	64	63	64	63	85	70
	3	57	62	56	52	58	56	64	62	78	72
	4	60	52	448	53	59	123	63	59	81	82
2×	1	57	57	61	63	59	61	72	70	100	89
	2	57	55	65	62	74	65	84	77	119	94
	3	50	56	56	58	66	77	72	78	109	117
	4	67	56	55	65	67	S‡	127	S	107	83
1×	1	53	55	70	69	119	70	90	91	127	159
	2	73	64	78	72	82	81	61	100	181	199
	3	65	62	77	80	89	83	107	97	293	483
	4	S	63	S	S	S	100	132	S	161	202
0.5×	1	70	77	153	143	129	134	145	148	S	S
	2	74	83	114	103	138	131	425	281	S	S
	3	75	69	113	118	137	151	1588	244	S	S
	4	74	94	S	139	149	S	S	400	S	S
0.25×	1	111	112	173	176	S	481	S	S	S	S
	2	136	176	295	274	S	S	S	S	S	S
	3	103	124	S	300	S	S	S	S	S	S
	4	S	118	S	S	S	S	S	S	S	S
0.125×	1	185	195	S	S	S	S	S	S	S	S
	2	253	588	S	S	S	S	S	S	S	S
	3	473	234	S	S	S	S	S	S	S	S
	4	S	S	S	S	S	S	S	S	S	S
.0625×	1	S	S	S	S	S	S	S	S	S	S
	2	S	S	S	S	S	S	S	S	S	S
	3	S	S	S	S	S	S	S	S	S	S
	4	S	S	S	S	S	S	S	S	S	S

* Dose expressed in milliliters.
† Rat A or B.
‡ S indicates survival.

toxins at a concentration of 4× (4 times the original). All concentrations of toxins were maintained continuously at 4 C. To make the next dilution, 40 ml of the pool (4×) were combined with 40 ml of diluent (triple distilled water). This provided 80 ml of toxins at a concentration of 2×. Further serial twofold dilutions were made to 0.0625× ($^1/_{16}$ × original concentration) and inoculated as planned.

Each investigator required 108 rats. These rats were caged in 54 consecutively numbered cages, each containing two animals. Each of the 54 treatment combinations was given to the two animals in one cage at the same time. The order of the treatments was randomized for each investigator. Response times-to-death, in minutes, were recorded for each rat and constituted the basic data.

RESULTS

The response times for animals are presented in Table 4. Although none of the controls appears in this table, none of either the first or second groups of control animals died. Some animals in the third control group challenged with 1.5 ml of toxins plus normal horse serum responded nearly the same as test animals challenged with 1.5 ml of toxins. The mean response times, in minutes, of these control animals by concentration of toxins are recorded in Table 5. The pattern of responses by the controls provided the needed assurance that the response of the test animals was specifically to the toxins of *B. anthracis*.

In spite of carefully controlled procedures and techniques, the results from one laboratory (technician 4) were so erratic that they were disregarded in any further analysis. Inspection of these data showed that technician 4 was the only one having reversal of results; i.e., a greater amount of toxins not killing and lesser amounts killing, or only one of the two test animals responding (except at doses eliciting a response above 300 min). These extremely variable results indicated that adequate controls on technique and environment were not maintained in this laboratory.

TABLE 5. *Mean response time by dose and concentrations of toxins*

Concn of toxin	Dose (ml)					Mean	Control*
	4	2	1.5	1	0.5		
4×	57.5	53.5	59.0	62.3	75.0	60.7	60.0
2×	55.2	60.7	66.4	75.2	105.1	69.0	70.0
1×	61.3	74.1	85.1	88.0	198.7	86.3	134.0
0.5×	74.4	121.6	136.3	247.0	8†	151.3	154.0
Mean	61.3	70.3	78.3	89.4	143.5	91.3	

* Control was 1.5 ml of toxins plus normal horse serum.

† All animals survived.

TABLE 6. *Analysis of variance of reciprocal response times*

Line no.	Effect	df	Sum of squares	Mean square	F*
1	Dose (D)	4	11.9272	2.9818	229.37†
2	Concentration (C)	3	16.5629	5.5210	424.69†
3	Technician (T)	2	0.1543	0.0772	5.94‡
4	D × C	12	1.7984	0.1499	11.53†
5	D × T	8	0.1485	0.0186	1.43
6	C × T	6	0.1180	0.0197	1.52
7	D × C × T	24	0.6452	0.0269	2.07
8	Error	60	0.7814	0.0130	
9	Total	119	32.1360		

* Error line 8 was used to test all effects.

† Approximate probabilities <0.001.

‡ Approximate probabilities <0.05.

The reciprocals of the response times were used for analysis, because reciprocal response times are nearly normally distributed with equal variances, whereas the untransformed response times are positively skewed with unequal variances (Finney, 1952). The analysis of variance on the reciprocal response times of 120 rats from the four highest concentrations and the five doses is shown in Table 6. From this analysis it was seen that both dose level and concentration had statistically significant effects on the response time of Fischer rats injected intravenously with anthrax toxins.

The analysis further showed an interaction between dose and concentration to be statistically significant. The mean response times by dose and concentration of toxins are given in Table 5. From the tabled means, it can be seen that the magnitude of this interaction is slight and had no practical significance in the further analysis and interpretation of these data.

The analysis also showed a statistically significant difference among technicians. Inspection of the data showed that mean response times for all rats responding for technicians 1, 2, and 3 were, respectively, 78, 83, and 83 min. This is a practically unimportant difference which we believe may in part be due to environmental factors, because genetic differences would be almost nil after 100 generations of inbreeding. The rats used by technician 1 came from the Beall colony, which was maintained in a different environment than the Klein colony animals used by the other two technicians. This raised the question as to the effect on this assay of Fischer rats procured from non-Detrick sources. To examine this effect,

TABLE 7. *Response times in minutes by supplier, operators, and rats*

Rat	Charles River Breeding Labs., Inc.		Microbiological Associates, Inc.	
	1*	2	1	2
1	83	87	91	85
2	88	84	84	89
3	86	86	91	89
4	83	82	88	85
5	91	84	89	92
6	87	89	88	84
7	94	88	90	101
8	88	83	92	87
9	87	83	96	102
10	91	86	77	87
11	105	83	89	93
12	94	85	94	7[illegible]
13	92	79	90	107
14	90	81	91	88
15	98	81	91	83
16	91	85	77	90
17	82	83	97	89
18	90	87	89	88
19	83	85	82	75
20	88	83	90	86
Harmonic *mean* response time	89.28	84.10	88.50	88.42

* Operator number.

commercially available Fischer rats obtained from two breeders were tested and found to be suitable for this assay. In this study, 20 Fischer 344 rats from each of two suppliers (Microbiological Associates, Inc., Bethesda, Md.; and Charles River Breeding Laboratories, Inc., Brookline, Mass.) were challenged in each of two laboratories. The response times of all 80 rats are reported in Table 7. No statistically significant difference in times of response for animals from the two suppliers was observed. A difference between the two operators and the interaction of operator × supplier was statistically significant at the 5% level. The mean response time of three of the four groups differed by less than 1 min, and the fourth group differed by approximately 5 min. This difference of about 5 min between these two groups could be caused by a difference of about seven units of toxins, which is well within the 95% confidence limits of an estimated potency. Thus, this difference, although statistically significant, was considered of no consequence concerning this assay.

A test to determine the storage characteristics of the reference toxins was conducted on a vial of the toxins which had been stored for 36 months. The test vial was reconstituted with 10 ml of triple-distilled water. Six rats were then challenged with these reconstituted toxins, according to the protocol described in this paper.

The estimate of potency from that test was 32.4 potency units per ml at the 1× concentration. This was essentially identical to the 32 units per ml set up in the definition. Therefore, it was concluded that the reference toxins had not changed with respect to potency during 36 months of storage.

Development of procedures for direct assay method. A potency assay should be based on dose expressed in terms of well-defined units. No such units have as yet been defined for anthrax toxins. Varying the amount of toxins by varying either dose or concentration would have a significant effect on the response time of rats; however, rats injected with 1 ml of toxins concentrated to 2× responded in about the same time (75 min) as rats injected with 2 ml of toxins concentrated at 1× (74 min). This relationship holds true for most other dose-by-concentration combinations for which the product of these two factors is a constant. If doses are converted into 0.5-ml units, and concentrations into 0.0625 units, then the doses and concentrations in Table 4 can be expressed as shown in Table 8.

The products of the marginal numbers in Table 8 for any two equivalent dose-by-concentration combinations are the same; thus, the product of two dose units and 32 concentration units gives 64 total potency units of toxins. Similarly, four dose units of 16 concentration units also contain 64 total potency units of toxins. We define the potency unit of anthrax toxins to be expressed as these products of dose by concentration of this particular lot of toxins.

If we were to carry the definition of a potency unit no further, then 1 ml of 1× concentration of any anthrax toxins, regardless of its actual effect in animals, would have 32 potency units.

TABLE 8. *Derivation of potency units of anthrax toxins*

Concn of toxins in 0.0625-fold units	Dose of toxins in 0.5 ml units				
	8	4	3	2	1
64	512	256	192	128	64
32	256	128	96	64	32
16	128	64	48	32	16
8	64	32	24	16	8
4	32	16	12	8	4
2	16	8	6	4	2
1	8	4	3	2	1

To standardize a potency unit, it is necessary to describe the association between the dose, in units, and the potency, in terms of a biological response to this particular lot of anthrax toxins. The potency of any other lot of toxins may then be measured by comparing the response to a known amount of the test toxins with the response to the same amount of the reference toxins.

These response characteristics were described as the dose-response relationship when measured doses of these toxins were injected intravenously into Fischer 344 rats. The challenged rats responded by dying at a time that is shown here to be highly dependent on the dose measured in potency units of these toxins.

The regression of mean reciprocal response times on the $\log_2$ of the potency units of anthrax toxins is shown in Fig. 1. The least squares line has the equation:

$$Y = b_0 + b_1X + b_2X^2 \qquad (1)$$

where Y is the mean reciprocal response time, X is the potency of anthrax toxins in $\log_2$ units, and the b values are regression coefficients computed from the data of this test. The values of the coefficients, their variances and covariances, are: $b_0 = -2.591$; $b_1 = 0.959$; $b_2 = -0.051$; $V(b_0) = 0.077121$; $V(b_1) = 0.009514$; $V(b_2) = 0.000068$; $V(b_0b_1) = -0.026902$; $V(b_0b_2) = 0.002238$; $V(b_1b_2) = -0.000800$. This regression line represents a basis upon which comparisons of potency of anthrax toxins can be made. Thus, test toxins can be assayed either indirectly against this curve, or directly with parallel assays of the reference toxins.

Development of procedures for indirect assay method. To use the responses of 120 rats to the reference toxins [for which the slope of response from the regression data (Fig. 1) has been calculated], we recommend use of the indirect method for standardizing unknown potencies of anthrax toxins. The regression was nearly linear for doses from 16 to 128 units, corresponding to response times from 240 to 65 min. Thus, although the concentration of test or unknown toxins is arbitrary, it should be of such concentration that 1 ml, injected intravenously, will kill a Fischer rat in not less than 65 min, nor more than 240 min.

To test the potency of test or unknown toxins, enough animals should be used so that the amount of variation in the final result, that can be attributed to the test rats, is at least no greater than the amount of variation contributed by the standard rats. Thus, at least six Fischer rats of 200 to 300 g from a suitable colony should be

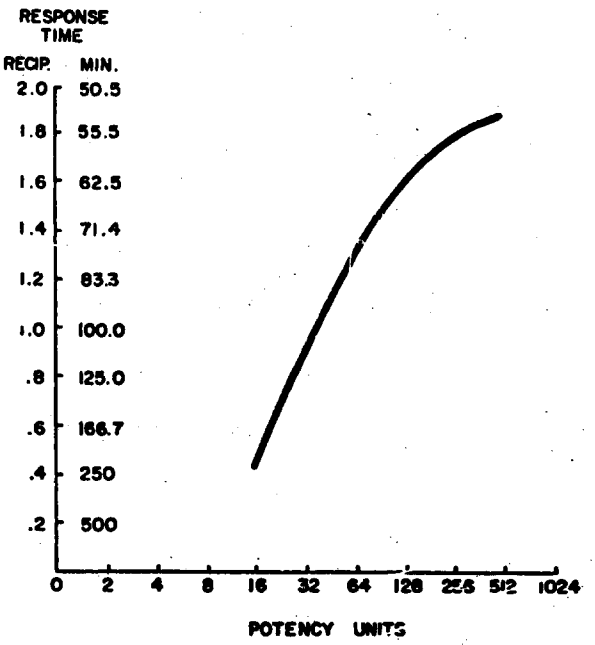

FIG. 1. *Regression of reciprocal response time of Fischer rats on log dose of anthrax toxins expressed in potency units.*

intravenously inoculated, three with 2 ml of the test toxins, and three with 1 ml.

The test is based on the mean reciprocal response times of the rats. (The rat response is very uniform; thus, any observed nonresponse must be considered the result of technique at some stage of the assay procedure.) This is simply the sum of reciprocal times-to-death of the rats in minutes (100/t) with the average time calculated. The reciprocal response times of the rats can be put in the following form:

Reference Toxins

$Y = 100/t$

1 ml		2 ml	
1.	____	4.	____
Rat 2.	____	5.	____
3.	____	6.	____
ΣY	____	ΣY	____
$\bar{Y} = R_1$	____	R_2	____

$R_1 + R_2 =$ ____

Test Toxins

$Y = 100/t$

1 ml		2 ml	
1.	____	4.	____
Rat 2.	____	5.	____
3.	____	6.	____
ΣY	____	ΣY	____
$\bar{Y} = T_1$	____	T_2	____

$T_1 + T_2 =$ ____

where R_1, R_2, T_1, and T_2 are mean reciprocal response times. This form for calculation can be used for either the direct or indirect assay method.

The estimate of the difference in potency (D) between the test toxins and the reference can be found as:

$$D = \frac{(T_1 + T_2) - (R_1 + R_2)}{2L} \quad (2)$$

where the letters T and R represent the mean reciprocal response times from the table above, and L is the average slope of the reference dose-response curve at the two dose levels used in the test. This average slope may be calculated as:

$$L = b_1 + b_2 (X_1 + X_2) \quad (3)$$

where X_1 and X_2 are the dose levels of the reference toxins (in $\log_2$ potency units) that were used in the test, and b_1 and b_2 are the estimates of the regression coefficients from equation 1. When the test is run using 1- and 2-ml doses of toxins, then $X_1 = 5$ and $X_2 = 6$. Under these conditions $R_1 = 0.92$, $R_2 = 1.34$ from equation 1, and $L = 0.3985$ from equation 3, so that equation 2 becomes:

$$D = \frac{(T_1 + T_2) - 2.26}{0.7970} \quad (4)$$

where the letter D represents the amount of difference between the test and reference toxins in terms of $\log_2$ potency units. If D is positive, then the test toxins are more potent than the reference, whereas, if D is negative, the test toxins are less potent than the reference. The reference toxins have a potency of 5 $\log_2$ units per ml at a concentration of 1×; thus, the potency (P) of the test toxins in $\log_2$ units at the concentration tested will be found as:

$$P = 5 + D \quad (5)$$

To find the number of potency units per ml of the test toxins, its potency needs to be converted from $\log_2$ units to $\log_{10}$ units. The conversion formula is:

$$\log_{10} P = \log_2 P \log_{10} 2$$

The value of P in units is found by looking up the antilog of this product. This value will be the number of potency units per milliliter of the test toxins at the concentration tested.

Estimation of variance. There is variation inherent in this assay system in addition to the variation between samples of toxins. Thus, the single estimates of the potency of any particular sample of an unknown toxin should be bounded by confident limits. To determine these limits it is necessary to calculate the variance (V) of the estimate D of the $\log_2$ of the difference in potency between the test and the reference. The variance of the estimate D will depend on the variances of the observed response times and of the regression.

If we express D as N/G where

$$N = (T_1 + T_2) - (R_1 + R_2) \quad (6)$$

and

$$G = 2L$$

then the variance of D can be expressed as:

$$V(D) = \frac{1}{4L^2} \{V(N) + D^2 V(G)\} \quad (7)$$

which will apply, because N and G are estimated from independent observations (Finney, 1952). The four mean reciprocal response times are stochastically independent; thus, the estimate of $V(N)$ can be expressed as:

$$V(N) = V(R_1) + V(R_2) + V(T_1) + V(T_2) \quad (8)$$

where $V(T_1)$ and $V(T_2)$ are obtained directly from the data of the test, and $V(R_1)$ and $V(R_2)$ are calculated from the regression line as:

$$V(R_i) = V(\bar{Y}) + (X_i - \bar{X})^2 V(b_1) + (X_i^2 - \bar{X}^2)^2 V(b_2) \quad (9)$$

The variance of G is given by the equation:

$$V(G) = 4\{V(b_1) + (X_1 + X_2)^2 V(b_2) + (X_1 + X_2) V(b_1 b_2)\} \quad (10)$$

When the test is run using 1- and 2-ml doses of toxins, then $X_1 = 5$ and $X_2 = 6$. Under these conditions:

$$V(R_1) = 0.0134,\ V(R_2) = 0.0018$$

and

$$V(G) = 0.0355$$

so that:

$$V(D) = \frac{1}{0.6352} \{V(N) + 0.0355 D^2\} \quad (11)$$

and:

$$V(N) = 0.0134 + 0.0018 + V(T_1) + V(T_2) \quad (12)$$

Example. A sample of toxins of unknown potency was tested in this laboratory. It was known to kill Fischer rats in slightly more than 90 min when injected intravenously in doses of 1 ml at a concentration of 1×. The response of the unknown toxins was compared with the response curve described by equation 1. Each of three

Fischer rats was injected with 1 ml of the test toxins, and their reciprocal response times in minutes were recorded (Fig. 2). Three other Fischer rats were each injected intravenously with 2 ml of the test toxins. Their reciprocal response times were also recorded (Fig. 2). From these six reciprocal response times, values of T_1 and T_2 were calculated. Corresponding values of R_1 and R_2 were obtained from the regression line by substituting, respectively, the values 5 and 6 for X in equation 1. The value of L was calculated from equation 3 by use of the values 5 and 6 for

Reference Toxin Y = 100/t	1 ml.	2 ml.
Rat 1		
Rat 2		
Rat 3		
$\sum Y$		
$\bar{Y} = R_i$	0.92	1.34
$R_1 + R_2$	2.26	
$\sum Y^2$		
$V(R_i)$	.0134	.0018

Test Toxin Y = 100/t	1 ml.	2 ml.
Rat 1	1.39	1.67
Rat 2	1.25	1.56
Rat 3	1.15	1.59
$\sum Y$	3.79	4.82
$\bar{Y} = T_i$	1.26	1.61
$T_1 + T_2$	2.87	
$\sum Y^2$	4.8171	7.7506
$V(T_i)$	.0048	.0011

$b_0 = -2.5912$
$b_1 = .9592$
$b_2 = -.0510$

$V(b_0) = .07712089$
$V(b_1) = .00951355$
$V(b_2) = .00006804$
$V(b_1 b_2) = -.000800$

$L = b_1 + b_2(x_1 + x_2)$

$x_1 = 5$ $x_2 = 6$ $(x_1 + x_2) = 11$ $(x_1 + x_2)^2 = 121$

$b_1 = 0.9592$

$b_2(x_1 + x_2) = 0.5607$

$L = 0.3985$

$2L = 0.7970$

$4L^2 = 0.6352$

$D = \dfrac{(T_1 + T_2) - (R_1 + R_2)}{2L} = \dfrac{2.87 - 2.26}{0.7970} = 0.78$

$D^2 = 0.6084$

$\text{Log}_2 P = 5 \pm D = 5$

$\text{Log}_{10} P = 0.301 \times 5.78 = 1.74$ $P =$ **55.0 U/ml**

$V(G) = 4\{V(b_1) + (x_1 + x_2)^2 V(b_2) + (x_1 + x_2) V(b_1 b_2)\} = 0.0355$

$V(N) = V(R_1) + V(R_2) + V(T_1) + V(T_2) = 0.0211$

$V(D) = \dfrac{1}{4L^2}\{V(N) + D^2 V(G)\} = 0.0672$

$SE(D) = 0.26$

$UL(D) = 1.30$ Log_{10} $UL(P) = 1.90$ $UL(P) = 79.4$

$LL(D) = 0.26$ Log_{10} $LL(P) = 1.58$ $LL(P) = 38.0$

FIG. 2. *Calculation form for potency of anthrax toxins.*

X_1 and X_2. The values 5 and 6 were used in these two cases, because they are the $\log_2$ of the number of units in 1 and 2 ml of the reference toxins.

The value of D was calculated by substituting the previously calculated values of R_1, R_2, T_1, T_2, and L in equation 2. This vaue of D was found to be 0.78. This indicates that the test toxins were 0.78 $\log_2$ unit more potent than the reference. A 1-ml amount of the reference toxins contains 5 $\log_2$ units, so the test toxins must contain 5.78 $\log_2$ units. Thus, the test toxins have 55.0 potency units per ml at the concentration tested (5.78 × .301 = 1.73978 $\log_{10}$ units).

The formulas for calculating the variance of the estimate D of the $\log_2$ of the difference in potency between the test and the reference are described above as equations 6 through 10. These calculations were made in this example, and it was found that SE (D) = 0.26. Using normal theory, the 95% confidence limits of D become $UL(D)$ = 1.30, and $LL(D)$ = 0.26. From these the 95% confidence limits of P were calculated as $UL(P)$ = 79.4 units per ml, and $LL(P)$ = 38.0 units per ml.

Discussion

Anthrax toxins are composed of at least three factors, I, II, and III, by the classification of Stanley and Smith (1961, 1963) or, respectively, edema factor, protective antigen, and lethal factor according to Beall et al. (1962). Both in vitro-produced toxins, as used in this report, and in vivo toxins, as reported by Klein et al. (1963), may be quantitated accurately. The procedure further provides an effective reference for quantitating natural resistance or relative immunity as described by Klein et al. (1963), because the absolute dose of toxins required to elicit a given response will bear a definite relationship to host resistance or susceptibility.

The biological activities of these compounds are numerous, and it is likely that some responses are still to be discovered. The problem of evaluating activity and mode of action of compounds which have a synergistic biological action is more difficult than for "single compounds." Quantitation, therefore, is important to allow the work of various investigators to be related more exactly to each other. The Fischer 344 rats are commercially available, and reference anthrax toxins will be provided for responsible investigators who desire to work with this material for use in establishing units. The methods used in this standardization of these toxins may be appropriate to the standardization of other biologically active toxins.

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